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Growth of acid fast L forms from the blood of patients with sarcoidosis

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Abstract

***Background* – Acid fast cell wall deficient forms (CWDF) of bacteria have been grown from blood, bronchial washings, and ocular anterior chamber fluid from patients with sarcoidosis. A monoclonal antibody raised against *Mycobacterium tuberculosis* whole cell antigen ($H_{37}RV$) was used to characterise further CWDF grown from the blood of patients with sarcoidosis.**

***Methods* – Blood from 20 patients with active sarcoidosis and from 20 controls was cultured using methods favourable for the growth of CWDF. Isolates were further characterised by indirect fluorescent antibody analysis using a monoclonal antibody highly reactive with *M tuberculosis*.**

***Results* – CWDF were grown from the blood of 19 of 20 subjects with sarcoidosis. All isolates stained positively with the monoclonal antibody and with a modified Kinyoun stain. No organisms were grown from the blood of controls.**

***Conclusions* – These data demonstrate that CWDF can be grown from the blood of nearly all patients with active sarcoidosis. The results confirm that the organisms are mycobacterial in origin and are similar, if not identical, to *M tuberculosis*. Their role in the pathogenesis of sarcoidosis is unknown.**

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Sarcoidosis is a multisystem disorder characterised by the presence of numerous non-caseating granulomas in many organs including lung, lymph nodes, spleen, liver, and skin. Although pathogenesis of the entity remains unclear despite intensive research efforts for nearly 90 years, cumulative findings strongly implicate mycobacteria as the causative factor. Acid fast bacilli have been detected in lymph nodes and lung tissue of patients with sarcoidosis[1–7] that appear to be mycobacterial cell wall deficient forms (CWDF).[8–11] Antibodies against unknown mycobacterial antigens have also been detected in the serum of most patients with sarcoidosis.[12] Increased numbers of γδ T lymphocytes, which proliferate in response to mycobacterial antigens, are present in peripheral blood samples from patients with sarcoidosis, with expansion of the Vδ2 + subset occurring in both sarcoidosis and tuberculosis.[13–16] Mycobacterial nucleic acid components have been found in bronchoalveolar lavage samples, lung tissue, spleen, and lymph nodes from patients with sarcoidosis.[17–20]

In addition, acid fast CWDF have been grown from the blood, bronchial washings, and ocular anterior chamber fluid from patients with sarcoidosis.[11 21–24] Specifically, Judge[22] and Mattman[23] grew CWDF from 28 of 29 subjects with sarcoidosis. However, in none of these studies was it clearly shown that organisms isolated by culture were of mycobacterial origin. In this study we have therefore used a monoclonal antibody raised against *Mycobacterium tuberculosis* $H_{37}RV$ whole cell antigen[25] to characterise further CWDF grown from the blood of patients with sarcoidosis.

Methods

SUBJECT SELECTION

Subjects were recruited from the Veterans Administration Medical Center, Hampton, Virginia. Blood samples were obtained from subjects with clinical, radiographic, and biopsy evidence of active sarcoidosis and whose sputum was acid fast smear and culture negative for tuberculosis.[26] All subjects had lung disease and, in addition, three had skin disease, one joint disease, and one central nervous system disease. Seven were receiving systemic corticosteroids. The duration of the disease ranged from 0 to 8 years. Blood samples were also obtained from healthy individuals, none of whom were known to be infected with the AIDS virus or to have a history of tuberculosis. All control individuals were skin test negative for purified protein derivative (PPD) and none had received Bacillus Calmette Guerin (BCG) vaccine. All subjects gave informed consent and the studies were approved by the Institutional Review Board.

ASSESSMENT OF MONOCLONAL ANTIBODY SPECIFICITY

A mouse monoclonal antibody was developed against *M tuberculosis* $H_{37}RV$ whole cell antigen using the Kohler-Milstein technique.[27] One gram of wet cell pellet of bacteria was suspended in 10 ml of sterile distilled water and inactivated by flowing steam (80–88°C) in an autoclave for one hour. The concentration of inactivated cells was adjusted to an absorbance of 0·15 in a 10 × 75 mm cuvette at 450 nm with a Coleman Junior IIA linear absorbance spectrophometer (Model 6/20A, Perkins Elmer Coleman Instruments Division); 160 μl of selected cell suspension was added to microtitre plates with Immunolon removal well strips (Dynatech Laboratories, Alexandria, Virginia,

Reactivity of anti-$H_{37}RV$ hybridoma antibody with different species of mycobacteria and non-acid fast bacteria (ELISA) expressed as optical density

Heated antigen	*Result*
$H_{37}RV$	>2·5
BCG	>2·5
$H_{37}RA$	0·81
M kansasii	0·00
M intracellulare	0·10
M scrofulaceum	0·07
M chelonae	0·00
M fortuitum	0·08
M avium	0·11
Pseudomonas aeruginosa	0·00
Escherichia coli	0·00
Klebsiella pneumoniae	0·00
Staphylococcus aureus	0·00
Streptococcus pyogenes	0·00

USA). Plates with cell suspensions were centrifuged at 1200 rpm for 10 minutes, the wells were aspirated, washed with a Tween 80 (0·05%) saline (0·85%) wash solution, and filled with ethylene glycol for five minutes to fix antigen to the plastic wells. Ethylene glycol was aspirated, the wells washed, and the plates were used or stored at 4°C for enzyme linked immunosorbent assay (ELISA) procedures. The $H_{37}RV$ monoclonal antibody was added to selected antigen wells, incubated at 37°C for one hour, aspirated, and washed. This was followed by addition of a goat antimouse alkaline phosphatase conjugate (Sigma Chemical Co., St Louis, Missouri, USA), incubation, and a wash procedure. Finally, 160 µl of alkaline phosphatase substrate (A-5153, Sigma Chemical Co.) was added to appropriate wells for incubation at room temperature for 30 minutes before recording colour development at 410 nm with a Dynatech ELISA minireader (Alexandria, Virginia, USA). As shown in the table, there was high absorbance with the $H_{37}RV$, BCG, and $H_{37}RA$ antigens, minimal absorbance with atypical mycobacterial antigens, and no absorbance with either Gram positive or Gram negative antigens.

BLOOD CULTURE ANALYSIS FOR L FORMS OF MYCOBACTERIA

Veal infusion broth (Difco Laboratories Inc, Detroit, Michigan, USA) and glycerol (2%, Fisher Scientific, Springfield, New Jersey, USA) were mixed and adjusted to a pH of 5·5 and combined with Noble agar (0·09%, Difco Laboratories) to make a modified veal infusion medium which was dispensed in 19 ml aliquots in glass screw cap tubes. A 20% solution of yeast extract (No. 0127, Difco Laboratories) was prepared and dispensed in 10 ml aliquots in separate glass screw cap tubes. The veal infusion medium and yeast extract were sterilised in an autoclave at 15 lbs (121°) for 15 minutes. Immediately before inoculation of the veal infusion medium with subjects' blood, 1 ml of the fresh yeast extract (20%) was added aseptically to 19 ml of the veal infusion medium to prepare veal infusion medium + yeast extract 1%.

Polyvinylpropylene iodine was used to clean the subject's skin and rubber stopper of a vacutainer tube containing EDTA. Approximately 2 ml of blood (for a 10% inoculum) was drawn into a sterile Pasteur pipette and transferred to a tube containing veal infusion medium + yeast extract. The inoculum was then mixed and randomly distributed throughout the medium and incubated at 36°C. At 48 hours 10 ml of growth was transferred to a glass screw cap tube, 0·1 ml of xylene was added, and the mixture was shaken by hand for five minutes. It was then allowed to stand in a vertical position for 10 minutes. The top portion of the xylene/medium emulsion was removed using a Pasteur pipette and transferred to glass slides for Gram stain, Kinyoun stain, and indirect fluorescent antibody analysis. To detect acid fast organisms, dried smears of growth from the blood cultures were fixed for three minutes using methanol. The methanol was then decanted and the smears were allowed to air dry. The primary stain, Kinyoun's carbol fuchsin, was used to flood the slides and stain the smears for five minutes. The primary stain was modified by adding 0·1 ml of 5% sodium bicarbonate to 9·9 ml of primary Kinyoun stain (HarleCo, Gibbston, New Jersey, USA), mixing, and filtering (no. 2 filter paper) just before use. After the primary stain was decanted the slides were flooded with 3% HCl in 95% ethanol (decolouriser) and rocked on a staining rack for one minute to remove unbound stain. After decolourisation the slides were gently washed in cold water and drained. The slides were then flooded with the counterstain metanil yellow (0·05% aqueous solution, HarleCo) for one minute, decanted, and allowed to air dry without blotting. The stained smears were placed in a slide holder at >45° for draining and drying. All washing procedures were performed with cold water from a beaker because forcibly applied water removes the loosely adherent L colonies. The smears were then examined under 20 × magnification to locate acid fast microcolonies. Their typical morphology was confirmed by examination under oil immersion (1000 ×).

Mycobacterial L forms were identified using the mouse monoclonal antibody raised again *M tuberculosis* $H_{37}RV$ whole cell antigen. Smears were fixed as described for the Kinyoun stain. The dried fixed smear was then rehydrated by flooding the slide with 0·01 M phosphate buffered saline (PBS), pH 7·6. After decanting the excess saline, the mouse anti-$H_{37}RV$ monoclonal antibody diluted in 0·01 M PBS, pH 7·6, was added to the smears which were incubated in a moist chamber at 37°C for 30 minutes. After incubation the monoclonal antibody was decanted and the smears were gently washed in cold PBS, pH 7·6, to remove unbound monoclonal antibody. The washing process was followed by the addition of goat antimouse IgG fluorescein isothiocyanate (FITC) conjugate (Sigma) to the smears for 20 minutes in a dark moist chamber at 37°C. After incubation, unbound conjugate was washed from the smears and glycerol/PBS mounting fluid (10 ml glycerol + 1 ml of PBS, pH 9·0) was added. After applying a glass cover slip the slides were examined with a Leitz Laborlux D fluorescence microscrope (Ernst Leitz Wetzlar GmbH, West Germany).

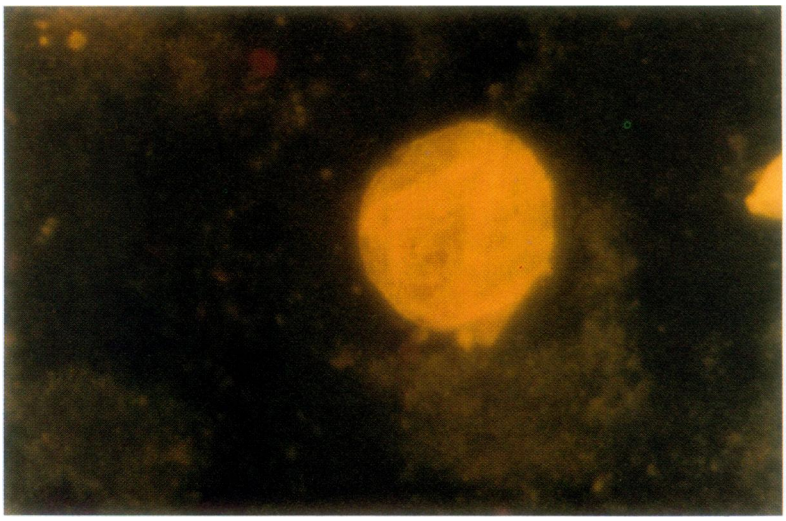

Figure 1 Photomicrograph showing cell wall deficient forms (CWDF) grown from the blood of a patient with active sarcoidosis using the Mycobacterium tuberculosis $H_{37}RV$ monocloncal antibody. A large yellow fluorescing L body is seen in the centre, and small yellow fluorescing microcolonies (× 400) are seen elsewhere.

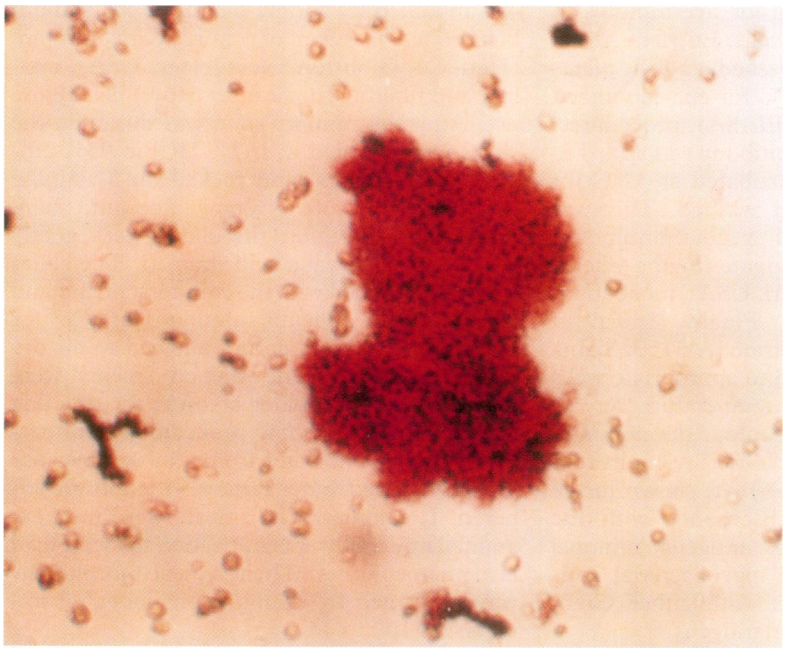

Figure 2 Photomicrograph showing cell wall deficient forms (CWDF) grown from the blood of a patient with active sarcoidosis. Using a modified Kinyoun stain, acid fast microcolonies are seen in the centre and red cells are seen in the background (× 200). The microcolonies are granular in appearance and red in colour (acid fast).

Results

A total of 40 subjects were studied. One group consisted of 20 subjects with documented sarcoidosis, and the second group consisted of 20 controls. During the study the two investigators (AJ and LHM) who performed the indirect fluorescent antibody analysis and modified Kinyoun stains were blinded to the final diagnosis. Subsequent decoding revealed that cell wall deficient bacteria grew from the blood of 19 of 20 subjects (95%) with sarcoidosis. All organisms were reactive with the $H_{37}RV$ monoclonal antibody, with obvious fluorescing microcolonies and L bodies (fig 1). All smears also stained positively with the modified Kinyoun stain (fig 2). No CWDF were detected in the blood of healthy controls by either indirect fluorescent antibody analysis or modified Kinyoun stain.

Discussion

Acid fast organisms were grown successfully from the blood of 19 of 20 patients with active sarcoidosis, whereas no organisms were grown from the blood of 20 controls. Findings of variable size, predominantly coccoid forms, larger L forms, and short acid fast rods suggested that the organisms were CWDF of mycobacteria. It has previously been established that these organisms grow a few millimetres below the surface in semisolid broth under microaerophilic conditions, but are unable to multiply under strictly anaerobic conditions.[23] The organisms do not depend on hypertonicity, as is true for many CWDF, and growth within red cells is a common characteristic. However, unlike CWDF grown from the blood of patients with tuberculosis, which revert to their wild type in vivo (intact cell wall) in the presence of antibiotics, the organisms grown from the blood of patients with sarcoidosis do not revert to a wild type under any culture condition. Our findings confirm previous observations that a CWDF can be grown from the blood of nearly all patients with sarcoidosis.[21 22]

The acid fast organisms grown from the blood of patients with sarcoidosis also stained positively with the $H_{37}RV$ monoclonal antibody which was raised against *M tuberculosis* $H_{37}RV$ whole cell antigen.[25] The antibody is weakly reactive to mycobacteria other than *M tuberculosis* and does not react with Gram negative or Gram positive bacteria. Our findings therefore confirm that CWDF isolated from patients with sarcoidosis belong to the mycobacterial family.

The fact that most patients with sarcoidosis were chronically colonised with a mycobacterial organism does not necessarily imply that the organism is the cause of the disease. However, in a detailed animal study Judge[22] observed that granulomatous lesions developed in many organs including the lung, liver, spleen, kidney, and eye in mice, gerbils, and guinea pigs following intraperitoneal instillation of CWDF grown from the blood of patients with sarcoidosis. Histological changes occurred as early as 19 days after injection and were more widespread in animals simultaneously given cortisone. Organisms identical to those injected were recovered by culture from the blood and body tissues of the animals. Similarly, Barth *et al*[11] induced "non-consolidated nodularity" in the lungs of mice inoculated with cortisone and acid fast CWDF grown from anterior chamber fluid from the eye of a patient with sarcoidosis. In an extensive series of studies by Mitchell *et al*[28] it was shown that, although mycobacterial organisms were not specifically identified, homogenates of human sarcoidosis tissue injected into the foot pads of mice caused the slow development of widely disseminated granulomas in lymph nodes, lung, liver, spleen, and muscle, that were associated with the development of a positive Kveim reaction. Granulomatous changes were transmitted through four consecutive animal passages.

In contrast, other investigators have failed to find evidence implicating mycobacteria as causative factors in sarcoidosis. Specifically, in several studies using fluorescence and electron

microscopy or varied culture methods, no evidence of mycobacteria or mycobacterial fragments was found in granulomatous tissue from patients with sarcoidosis.[29 30] In addition, although Taub *et al*[31] were able to induce granulomas in the foot pads of mice with homogenates of lymph nodes from patients with sarcoidosis, the changes were restricted to the site of injection and no abnormalities developed in lymph nodes, liver, spleen, or lung, and there was no response to Kveim antigen. However, since the samples were homogenised prior to injection, the growth of organisms may have been altered. Interestingly, no granulomas developed following freezing and thawing of homogenates. It has previously been reported that CWDF (spheroblasts) of *M tuberculosis* caused no pathological changes when instilled intratracheally into guinea pigs, even though the organisms persisted in the lungs for prolonged periods of time.[32]

In general, recent molecular biology studies have found a link between mycobacteria and sarcoidosis. Saboor *et al*[17] found *M tuberculosis* DNA in 50% of patients with sarcoidosis and non-tuberculosis mycobacterial DNA in a further 20%, using a complex specific insertion sequence IS986/IS6110 to detect DNA from *M tuberculosis* complex bacteria and the conserved sequences of the mycobacterial groEL gene to detect DNA from mycobacteria other than *M tuberculosis*. The false positive polymerase chain reaction (PCR) rate for *M tuberculosis* was 9%. In a similar study using the same primers the authors found *M tuberculosis* DNA in granulomatous tissue from seven of 16 patients with sarcoidosis.[18] Mitchell *et al*[19] used liquid phase DNA/RNA hybridisation with a DNA probe specific for the rRNA of the *M tuberculosis* complex and found that hybridisation with spleen tissue from patients with sarcoidosis was 4·8 times higher than that with normal spleens. By contrast, Bocart *et al*[33] found *M tuberculosis* DNA in tissue samples from only two of 18 patients with sarcoidosis using several oligonucleotide primers including the IS6110 insertion element. However, no patients with proven tuberculosis were included as positive controls. With the use of paraffin embedded tissue samples and PCR amplification, Popper *et al* found strong signals for mycobacterial DNA in two of 15 samples from patients with sarcoidosis,[20] whereas Ghossein *et al*[34] failed to find mycobacterial DNA in samples from 10 patients using similar techniques.

In this study we have shown that organisms grown from the blood of patients with sarcoidosis are mycobacterial in origin. It is anticipated that specific DNA sequencing will be necessary to determine whether these organisms are CWDF of *M tuberculosis*, a closely related atypical organism, or a unique species.

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